SECTION V

REVISED PROPOSED REFERENCE SUBSTANCES FOR OPTIMIZATION/VALIDATION STUDIES

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1.0 INTRODUCTION

On November 1, 2004, NICEATM released draft BRDs on the current status of four *in vitro* test methods for detecting ocular corrosives and severe irritants (BCOP, HET-CAM, IRE, ICE). Included in each BRD was a list of proposed reference substances for the optimization and/or validation of *in vitro* tests to detect ocular corrosives and severe irritants (available electronically at http://iccvam.niehs.nih.gov/methods/ocudocs/ocu_brd.htm). The proposed reference substances are intended to:

- represent the range of ocular responses (i.e., corrosive/severe irritant; nonsevere irritant/noncorrosive) that the test method is expected to be capable of predicting
- represent the range of chemical/product classes and physicochemical properties (e.g., solid, liquid) that the test method is expected to be capable of testing
- represent the range of known or anticipated mechanisms or modes of action for severe/irreversible ocular irritation or corrosion
- have been generated by high-quality *in vivo* rabbit eye test method studies following Organization for Economic Cooperation and Development (OECD) Test Guideline (TG) 405 (OECD [1987]) and preferably conducted in compliance with Good Laboratory Practices (GLP) guidelines (OECD [1998]; EPA [2004a, 2004b]; FDA [2004])
- have a well-defined chemical composition
- be tested at a defined concentration and at a defined purity[1]
- be readily available

On January 11-12, 2005, ICCVAM convened an Expert Panel to independently consider this list of proposed reference substances; the Expert Panel concluded that the list of proposed substances is fairly comprehensive in that the three major groups of products to which the eye is exposed (i.e., industrial chemicals, pharmaceuticals, cosmetics) are represented and that, in general, individual substances were appropriately chosen. In addition, the Expert Panel suggested several changes to the list of proposed reference substances (see the Expert Panel Report, *Evaluation of the Current Validation Status of In Vitro Test Methods for Identifying Ocular Corrosives and Severe Irritants;* this report can be obtained by contacting NICEATM or electronically from http://iccvam.niehs.nih.gov/methods/eyeirrit.htm). In response to their recommendations, a revised list of proposed reference substances has been developed that includes the following changes:

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[1]Information on purity and the concentration tested were not available for all substances included in the NICEATM *in vivo* rabbit eye test results database. A decision was made to exclude nonsevere irritants (i.e., GHS Category 2A or 2B irritants) or non-irritants but not corrosive/severe irritants (i.e., GHS Category 1) that lacked concentration data from consideration as proposed reference substances. GHS category 1 substances were included because testing at a potentially higher concentration would not likely alter their classification as a GHS Category 1 substance although it might alter the criteria by which they were classified as an ocular corrosive/severe irritant. Where information on purity was lacking, an assumption was made that testing would have been conducted with a relatively pure substance. For substances included because they cause severe ocular effects in humans but lacked appropriate *in vivo* rabbit eye test data, information on concentration and purity were not available.

- The number of inorganic substances has been increased. In addition to the two inorganic substances in the original list (potassium tetrafluoroborate, sodium perborate tetrahydrate), 11 additional inorganic substances (aluminum chloride, antimony oxide, lime, magnesium hydroxide, nitric acid, silver nitrate, sodium hydrogen difluoride, sodium hydrogen sulfate, sulfuric acid, zinc chloride) have been added. Also, as recommended by the Expert Panel, many of the additional inorganics are used in consumer products.
- Substances that are known human ocular corrosives or severe irritants, even in the absence of high quality Draize rabbit eye test data, have been added. Based on human data only, ten such substances were added; these are ammonia, chloroform, lime, magnesium hydroxide, nitric acid, potassium hydroxide, silver nitrate, sodium hydrogen difluoride, sulfuric acid, and zinc chloride.
- All 12 formulations in the original proposed list have been excluded.
- The number of surfactants has been reduced from 12 to seven.

In addition,

- the source of the Draize rabbit eye test data has been provided for each proposed reference substance
- where applicable and to the extent possible, within a chemical class, substances of lower, medium and higher molecular weight have been included (the molecular weight of each proposed substance is now provided)
- information is provided on whether each proposed reference substance has been tested in the proposed version of BCOP, HET-CAM, ICE, and IRE test methods

In addition to these recommendations, the Expert Panel commented that the total number of proposed reference substances (i.e., 89) was large, and suggested that an appropriate number of specific substances should be selected that would be considered optimal for optimization and validation studies. No specific guidance was provided as to how to determine the appropriate number of reference substances. The Expert Panel suggested that a two-stage study design could be employed for validation studies, where a small subset of the reference substances could be used to make an initial assessment of accuracy and/or reliability. If the test method was considered promising after this initial assessment, then a larger number of substances could be used to further characterize test method accuracy and/or reliability.

The number of substances needed to adequately evaluate the accuracy of an alternative test method will vary with the proposed use and mechanistic basis of the test method. Based purely on a statistical evaluation, several hundred substances could potentially be required to evaluate accuracy with a reasonably high level of confidence, even when any effects of differential potencies among the reference substances are assumed to be negligible[2]. Generally, the (1) greater the range of possible responses (in terms of potency) that the test

[2] A formal statistical evaluation is being conducted by NICEATM to estimate the appropriate number of substances to use in evaluating the accuracy of an *in vitro* ocular irritancy test method.

method is expected to be capable of measuring or predicting, (2) greater the diversity of known or anticipated mechanisms or modes of action that are involved in the toxic response, and (3) greater the number of chemical/product classes and physicochemical properties that the test method is expected to be capable of testing, the greater the number of reference substances that will be needed to adequately demonstrate the validity of an alternative test method. For the detection of ocular corrosives and severe irritants, the list of reference substances needs to include substances that:

- induce very severe responses within a relatively short time period, as well as those where the toxic response is delayed
- adversely affect the cornea, iris, and/or conjunctiva
- induce persistent versus non-persistent lesions (when assessed at 21 days post treatment)
- represent diverse chemical classes and physicochemical properties

To meet these needs, the total number of substances in the list was increased from 89 to 122.

Clarification regarding the rules for classification of severe irritants was obtained subsequent to the release of the four BRDs. This resulted in changes to the hazard classification of a few of the substances included in the original list of proposed reference substances. For the original analysis, reversibility of ocular effects for the EU and UN GHS hazard classification systems was considered to be achieved if, by post-exposure day 21, the endpoint scores fell below the threshold that resulted in a test substance being classified as a severe irritant (EU [2001]; UN [2003]). The new information obtained indicated that reversibility of ocular effects is achieved only when all scores reach zero by post-exposure day 21. This change resulted in a few substances previously classified as nonsevere irritants now being classified as severe irritants.

The chemical classes assigned to each reference substance were revised based on the MeSH chemical classification system, an internationally recognized standardized classification scheme (see http://www.nlm.nih.gov/mesh and **Appendix B**). This resulted in some reference substances being re-classified into other chemical classes, which impacted on the number of reference substances in the various chemical classes.

Finally, additional *in vivo* rabbit ocular irritancy test results were obtained from several sources that expanded the number of potential candidate substances and which needed to be considered. Additional *in vivo* rabbit eye test data were received from[3]:

- Mr. Menk Prinsen (TNO Nutrition and Food Institute), for the 44 substances reported on in Prinsen (1996) and for 50 additional substances tested at TNO (Prinsen [2005])
- ZEBET, for the 144 substances that were described in Spielmann et al. (1996) (Spielmann and Liebsch [2005a])

[3] The efforts of these individuals, institutions, and organizations that provided additional data and/or information are gratefully acknowledged.

- Drs. Vanparys and Van Goethem for the 101 substances reported in the Gilleron et al. (1996, 1997) studies
- EPA for 43 substances in the Toxic Substances Control Act (TSCA) database not previously considered

To be considered a candidate reference substance, sufficient individual rabbit data to classify its ocular irritancy according to the GHS classification system (2003) was required, and the substance needed to be readily available. The revised list, as well as the draft list of proposed reference substances, was developed on the basis of preset selection criteria as outlined in Section 12 of each draft BRD. As a result of this additional data, the total number of commercially available candidate reference substances increased from 197 to 210 (see **Table V-1**).

2.0 REVISED LIST OF PROPOSED REFERENCE SUBSTANCES

The complete list of candidate substances from which the revised proposed list of reference substances was selected is provided in **Appendices V-A1** and **V-A2**, along with detailed information for each substance. In **Appendix V-A1**, the list is sorted by CASRN and then by substance concentration, which results in all tests conducted with the same substance being sorted together, regardless of the resulting GHS ocular hazard classification (UN [2003]). In **Appendix V-A2**, the list is sorted first by GHS ocular hazard classification and then by substance name. Proposed reference substances are bolded. An explanation as to why a GHS Category 1, 2A, or 2B substance was excluded from the list of proposed reference substances is provided in the comment column of this appendix. Selection of the 15 nonirritants was based on their ability, to the extent possible, to represent the range of nonirritating responses reported *in vivo* for treated rabbits and to match the chemical/product classes and physicochemical properties included among the corrosive or severely irritating substances proposed as reference substances. The range of nonirritating responses was determined by inspecting the extent to which the treated rabbits exhibited a response other than one that would result in the test substance being classified as an irritant (i.e., GHS Category 2A/2B or Category 1; see **Appendix A**).

The revised list of reference substances proposed for future optimization and validation studies of alternative test methods intended to detect ocular corrosives/severe irritants is provided in **Appendix V-B**; this list is sorted first by GHS ocular hazard classification and then by substance name. In **Appendices V-C** and **V-D**, the proposed reference substances are sorted by chemical class and by product class, respectively. The revised list includes 79 GHS Category 1 substances (10 of which were classified as severe irritants based on human data only), 28 GHS Category 2 substances (14 Category 2A substances, 13 GHS Category 2B substances, and one substance [Triton X-100] that induced a GHS Category 2A response in one study and a 2B response in another study when tested at a 5% concentration), and 15 nonirritants. These 122 substances cover 34 chemical classes and 24 product classes and include 79 substances tested in liquid form and 43 tested as solids. The number of substances per chemical class range from one for lactones, quinones, boron compounds, and amino acids to 22 for alcohols. For many of these chemical classes, the number of substances may be too few to adequately demonstrate the accuracy of a test method for that

specific chemical class. These numbers, however, reflect the maximum number of available substances for those chemical classes identified in the candidate list of reference substances (**Appendix V-A**). The large number of alcohols in this list reflects the fact that ICE, BCOP, and HET-CAM all currently demonstrate a low accuracy for such substances, as indicated in **Sections 2** and **3** of this addendum, respectively. Thus, a large number of reference alcohols is deemed useful for the further development of these test methods. Due to the fact that alcohols are relatively common substances for which there is considerable *in vivo* data, it proved possible to include alcohols distributed across the full range of ocular toxicity categories (i.e., 11 GHS Category 1, 4 GHS Category 2A, 4 GHS Category 2B, 3 nonirritants).

2.1 Performance Standards and Proficiency Substances

Following completion of the proposed validation studies, reference substances from this list can be selected for inclusion in performance standards and for proficiency testing. This list of proposed reference substances is intended to represent the minimum number of substances considered critical to an evaluation of the validity of alternative *in vitro* ocular irritancy test methods proposed for evaluating substances from a broad range of chemical and product classes. Subsets of substances from this list may be considered for:

- optimization of a test method protocol
- performance standard reference substances for use in the validation of test methods that are functionally and mechanistically similar to a validated ocular irritancy test method
- proficiency testing to ensure the competency of a laboratory in performing a validated ocular irritancy test method

In situations where a listed substance is unavailable, other substances of the same class for which high quality *in vivo* reference data are available could be used. Furthermore, if desired, additional substances representing other chemical or product classes and for which high quality *in vivo* rabbit eye reference data are available can be added to the minimum list of reference substances to more comprehensively evaluate the accuracy of a test method.

The database of substances from which this list of reference substances was developed includes representatives from each of the four ocular hazard classifications according to the GHS classification system (UN [2003]) (**Table V-1**). **Table V-1** also includes information on the range of molecular weights for the proposed substances in each GHS ocular hazard classification. The GHS Category 1 substances that are included in the list cover the entire range of responses that could result in a corrosive/severe irritant classification, based on both persistence and severity of the resulting lesion (**Table V-2**).

Table V-1. Distribution of Substances in the *In Vivo* Rabbit Eye Test Database and Molecular Weight Ranges of the Proposed Reference Substances, by GHS[1] Ocular Hazard Classification

Classification (GHS)	Number of Entries in the *In Vivo* Rabbit Eye Test Database[2] with a GHS Classification Revised/Original	Number of Candidate Substances (i.e., GHS-Classified Substances[3] Determined to be Commercially Available) Revised/Original[4]	Number of Proposed Reference Substances Revised/Original	Additional Substances Identified as Causing Severe Ocular Damage in Humans	Final Number of Proposed Reference Substances Revised/Original	Molecular Weight Range for Proposed Reference Substances
Category 1	220/123	93/48	69/48	10	79/48	30.0 – 546.8
Category 2A	62/24	17/11	15/11	-[5]	15/11	58.1 – 384.4
Category 2B	51/68	23/27	13/15	-	13/15	80.0 – 265.3
Nonirritant	497/277	77/111	15/15	-	15/15	86.2 – 1227.5
Total	**830/492**	**210/197**	**112/89**	**10**	**122/89**	**30.0 – 1227.5**

[1]GHS = Globally Harmonized System (UN [2003]).

[2]The complete database includes multiple entries for some substances, as well as formulations, coded substances, and substances that could not be classified according to the GHS ocular hazard classification system.

[3]"Substances" is defined as a unique entry (i.e., a single substance tested at a single concentration). The substances identified as causing severe ocular effects in humans are substances for which individual rabbit eye test results were not located. One substance (Triton X-100), when tested at 5%, induced a GHS Category 2A response in one study and a Category 2B response in another study; for purposes of classification in this table, Triton X-100 is classified as a Category 2A substance.

[4] The number of entries decreased for some GHS classification categories due to (1) the reclassification of some substances as GHS Category 1 irritants, based on the persistence of any lesion to day 21 post-treatment; (2) a reassessment of current commercial availability; and (3) collapsing multiple studies with the same substance tested at the same concentration into a single entry.

[5]"-" = not applicable.

Table V-2. NICEATM-Defined Subcategories for the Proposed GHS[1] Category 1 Reference Substances

Subcategory	Criteria for Classification as a GHS Category 1	# of Substances Revised/Original
0[2]	Not Classifiable	12/0
1	Positive response based on a persistent lesion involving the cornea, iris, and/or conjunctiva through to day 21 in at least one of three rabbits and not on severity	9/18
2	Positive response based on mean for first 3 days (CO[3] score >3 and <4 or IR[4] score >1.5) in at least two of three rabbits but lesions do not persist through day 21	4/4
3	Positive response based on mean for first 3 days (CO score >3 and <4 or IR score >1.5) in at least two of three rabbits and a persistent (>21 days) lesion in at least one rabbit	4/2
4	CO score = 4 at any time in at least one of three rabbits	50/24
	Total	**79/48**

[1]GHS = United Nations Globally Harmonized System (UN [2003]).
[2]Included are two GHS Category 1 substances that could not be subclassified because classification was based on an extreme response shortly after treatment in the only animal tested and 10 substances classified as GHS Category 1 irritants because they induced a severe ocular response in accidentally-exposed humans, and appropriate *in vivo* rabbit ocular irritancy test data was not located for these 10 substances.
[4]IR = iritis.

Because of their limited numbers, all of the commercially available GHS Category 2 substances with concentration data have been included in the list of proposed reference substances. As indicated in **Table V-3**, the current list of proposed reference substances covers a wide range of chemical classes and includes both solids and liquids. Substances were assigned into one or more chemical classes (see **Appendix B**). **Table V-4** summarizes the proposed reference substances by product class. All substances were assigned into one or more product classes by referencing the National Library of Medicine Hazards Substances Database (HSDB; see http://www.nlm.nih.gov/pubs/factsheets/hsdbfs.html); other information was obtained from Material Safety Data Sheets (MSDS) obtained from the commercial supplier.

Table V-3. Chemical Classes and Properties of Interest Represented Among the Proposed Reference Substances, According to GHS[1] Ocular Hazard Classification Category

Chemical Class[1]	Number of Candidate Substances	Number of Proposed Reference Substances	GHS Category 1[2]		GHS Category 2A	GHS Category 2B	GHS NI[3]
			Based on Human Data	Based on Rabbit Data			
Chemical Class[4,5]							
Acid (inorganic)	**2**	**2**	2(2)	-[6]	-	-	-
Acid (organic)	**20**	**17**	-	13(14)	2(2)	1(3)	1(1)
Acyl Halide	**3**	**3**	-	2(2)	1(1)	-	-
Alcohol	**30**	**22**	-	11(17)	4(6)	4(4)	3(10)
Aldehyde	**6**	**4**	-	2(2)	1(1)	1(2)	0(1)
Alkali	**3**	**3**	2(2)	1(1)	-	-	-
Amide	**2**	**2**	-	1(1)	-	1(1)	-
Amidine	**6**	**5**	-	4(5)	-	-	1(1)
Amine	**23**	**17**	-	14(18)	-	2(2)	1(3)
Amino Acid	**1**	**1**	-	1(1)	-	-	-
Boron Compound	**1**	**1**	-	1(1)	-	-	-
Ester	**30**	**15**	-	8(9)	3(3)	2(5)	2(15)
Ether	**22**	**11**	-	8(12)[7]	1(1)[7]	2(2)[7]	3(14)[7]
Heterocyclic Compound	**13**	**13**	-	9(13)[8]	2(2)[8]	2(2)	1(3)
Hydrocarbon (acyclic)	**7**	**1**	1(1)	-	-	-	0(6)
Hydrocarbon (cyclic)	**11**	**2**	-	-	1(1)	0(1)	1(9)
Hydrocarbon, Halogenated	**13**	**2**	-	-	-	-	2(13)
Isocyanate	**2**	**2**	-	2(2)	-	-	-
Ketone	**8**	**5**	-	-	2(2)	2(4)	1(2)
Lactone	**1**	**1**	-	-	1(1)	-	-
Nitrate	**2**	**2**	1(1)	-	-	1(1)	-
Nitrile	**3**	**3**	-	1(1)	1(1)	1(1)	-
Nitro Compound	**5**	**2**	-	2(2)	-	-(2)	-(1)
Onium Compound	**6**	**6**	-	5(8)[8]	1(1)[8]	1(1)	-(1)
Organophosphorus Compound	**3**	**2**	-	1(1)	1(1)	-	0(1)
Organosilicon Compound	**5**	**4**	-	3(3)	-	1(1)	-(1)
Phenol	**6**	**6**	-	5(7)	-	-	1(1)
Polycyclic Compound	**4**	**3**	-	2(3)	1(1)	-	-
Quinone	**1**	**1**	-	1(1)	-	-	-
Salt (inorganic)	**12**	**12**	7(7)	4(4)	-	-	1(1)
Salt (organic)	**13**	**12**	-	6(7)	-	2(4)	3(3)
Sulfur Compound (inorganic)	**1**	**1**	1(1)	-	-	-	-

Chemical Class[1]	Number of Candidate Substances	Number of Proposed Reference Substances	GHS Category 1[2]		GHS Category 2A	GHS Category 2B	GHS NI[3]
			Based on Human Data	Based on Rabbit Data			
Sulfur Compound (organic)	**15**	**9**	-	7(8)	-	-	2(8)
Urea Compound	**1**	**1**	-	-	-	1(1)	-
Total[9]	**281**	**193**	14(14)	116(143)	22(24)	24(37)	23(95)
Properties of Interest							
Liquid	**163**	**79**	6(6)	40(61)	14(16)[10]	9(13)	10(67)
Solid	**53**	**43**	4(4)	29(31)	1(1)	4(8)	5(9)

[1]Chemical Class=Based on the MeSH Medical Subject Heading. Available http://www.nlm.nih.gov/mesh; substances may be assigned into one or more chemical classes (see **Appendix B**).
[2]GHS = Globally Harmonized System (UN [2003]).
[3]NI = nonirritant.
[4]Numbers in parenthesis indicate the number of candidate substances for that GHS category.
[5]Substances were assigned into one or more chemical classes (see **Appendix B**).
[6]" – " indicates that there are no substances in this category.
[7]Triton X-100 classified as GHS Category 1, 2A/2B and NI.
[8]Cetylpyridinium bromide classified as GHS Category 1 and 2A.
[9]The total number is greater than the total number of proposed reference substances because some substances were assigned to more than one chemical class.
[10]Triton X-100, when tested at 5%, induced a GHS Category 2A response in one study and a Category 2B response in another study; for purposes of classification in this table, Triton X-100 is classified as a Category 2A substance.

Table V-4. Product Classes Represented Among the Proposed Reference Substances, According to GHS[1] Ocular Hazard Classification Category

Product Class	Total[2]	GHS Category 1		GHS Category[3] 2A	GHS Category 2B	GHS NI[4]
		Based on Human Data	Based on Rabbit Data			
Adjuvant, Solubilizer, Wetting Agent	**1**	-[4]	1	-	-	-
Anesthetic	**2**	1	1	-	-	-
Anti-Fungal	**6**	1	4	1	-	-
Anti-Infective	**12**	3	7[3]	3[3]	-	-
Battery Acid	**1**	1	-	-	-	-
Building Material	**2**	1	-	-	-	1
Caustic Agent	**2**	-	2	-	-	-
Chemical Intermediate	**43**	8	23	4	3	5
Cleaner or Cleaning Agent	**15**	6	6	1	1	1
Cosmetic Ingredients, & Perfumes	**11**	-	8	-	2	1
Fertilizers	**4**	4	-	-	-	-
Flame Retardant	**3**	2	1	-	-	-
Food Additives	**9**	2	4	1	1	1
Herbicides	**5**	2	2	1	-	-
Industrial Chemicals & Dyes	**46**	11	28	2	2	3
Laboratory Chemicals	**28**	3	16[3]	3[3]	3	4
Pesticide & Pesticide Intermediates	**17**	1	11	1	1	3
Pharmaceuticals & Pharmaceutical Intermediates	**29**	5	15	1	4	4
Polish	**1**	-	1	-	-	-
Preservative	**4**	2	1	1	-	-
Refrigerant	**1**	1	-	-	-	-
Solvent	**21**	1	8	8	3	1
Surfactants:	**7**	-	5[3]	2[3]	2[3]	2[3]
Anionic	**3**	-	1	-	1	1
Cationic	**2**	-	2[3]	1[3]	-	-
Nonionic	**2**	-	2[3]	1[3]	1[3]	1[3]
Veterinary Agent	**6**	2	4	-	-	-

[1]GHS = United Nations Globally Harmonized System (UN [2003]).

[2]All substances were assigned into one or more product classes by referencing the National Library of Medicine Hazards Substances Database (HSDB), other information was obtained from Material Safety Data Sheets (MSDS) obtained from the commercial supplier; therefore, the total number is greater than the total number of proposed reference substances.

[3]Some substances, when tested at different concentrations, were assigned a different GHS ocular hazard classification. For this table, these substances (Triton X-100 and cetylpyridinium bromide) appear in more than one GHS category column; thus the total numbers in these columns do not add up to the numbers of substances in the total column.

[4]" – " indicates that there are no substances in this category.

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